Volumetric Muscle Loss: Persistent Functional Deficits Beyond Frank Loss of Tissue

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Received 1 July 2014; accepted 8 August 2014
Published online 18 September 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22730

ABSTRACT: Open fracture is a common occurrence in civilian and military populations. Though great strides have been made in limb salvage efforts, persistent muscle strength deficits can contribute to a diminished limb function after the bone has healed. Over the past decade, a growing effort to establish therapies directed at de novo muscle regeneration has produced several therapeutic approaches. As this effort progresses and as therapies reach clinical testing, many questions remain regarding the pathophysiology of the volumetric loss of skeletal muscle. The current study demonstrates, in a rat "open fracture" model, that the volumetric loss of skeletal muscle results in persistent functional deficits that are dependent on muscle length and joint angle. Moreover, the injured muscle has an increased stiffness during passive stretch and a reduced functional excursion. A case study of a patient with an open type III tibia fracture resulting in volumetric muscle loss in the anterior and posterior compartment is also presented. Eighteen months after injury and tibia healing, persistent functional deficits are apparent with many of the same qualities demonstrated in the animal model. Muscle architectural adaptations likely underlie the altered intrinsic functional characteristics of the remaining musculature. Published 2014. This article is a U.S. Government work and is in the public domain in the USA. J Orthop Res 33:40–46, 2015.

Keywords: volumetric muscle loss; open fracture; soft tissue trauma; fibrosis; functional deficit

Open fracture often results in concomitant soft tissue injury. A variety of surgical procedures and biological interventions are available to improve bone healing, although therapies are limited primarily to physical rehabilitation for skeletal muscle healing. For instance, free or rotational flaps are placed in the sites of large skeletal muscle defects (i.e., volumetric muscle loss [VML][1]) concomitant with type III open tibia fracture.[2] Inasmuch as the intent of muscle flaps is to support bone healing, they offer little appreciable benefit towards muscle regeneration. Predictably, without a definitive therapy capable of regenerating ablated muscle tissue, persistent strength deficits secondary to VML can contribute to disability.[3] That is, skeletal muscle weakness and suboptimal limb function are generally considered part of the sequelae of musculoskeletal trauma.[1,4]

The gross removal of a portion of a muscle, as occurs with VML, promotes remodeling of the remaining musculature[5,6] that almost certainly changes the muscle's architecture (e.g., fiber length to muscle length ratio) and composition (e.g., increased collagen I content). Therefore it is expected that in addition to reductions in maximal torque[5,6] the active and passive length-tension characteristics of the injured muscle are also altered. The length-tension relationship of a muscle is fundamental to its performance in vivo and thus physical rehabilitation regimens are dependent on adaptations of this intrinsic muscle property. Moreover, as regenerative medicine and tissue engineering therapies progress to the clinic,[7] an improved understanding of the mechanical adaptation of VML injured muscle may improve product design and surgical use. Therefore, the purpose of this study was to characterize the impact of musculoskeletal trauma involving VML on rat TA muscle length-tension properties in situ and joint angle-torque properties in vivo. A case report of a patient with a type III open tibia fracture and VML is provided to further communicate the functional deficiencies of this condition.

METHODS

Experimental Design

Male Lewis rats were allotted to 1 of 4 experimental groups: Sham-operated, Osteotomy, VML injury or Osteotomy + VML injury. The Osteotomy + VML injury group was designed as a model of open fracture, in which the bone eventually heals but the muscle defect does not. The Osteotomy group only had tibia fracture, while the VML group only had TA muscle injury. And, the Sham-operated group underwent all surgical procedures, including blunt dissection of the soft tissue from the middle third of the tibia and insertion of tibia intramedullary pin, but did not have an osteotomy or VML created. In vivo functional analyzes of the TA muscle were performed at 4 or 8 weeks post-injury (*only at 4 weeks for Sham*) and in situ functional analyzes were performed on a subset of TA muscles 4 weeks post-injury. TA muscles were harvested at 4 and 8 weeks post-injury for histological and molecular analyzes. All animal procedures were approved by the Institutional Animal Care and Use Committee and were conducted in compliance with the Animal Welfare Act and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals.

Tibia Osteotomy

Rats were anesthetized and injected with sustained release buprenorphine-HCl (1.2 mg/kg SC; ~3 days release). The

Conflicts of Interest: None
Grant sponsor: Combat Casualty Care Research Program.
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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JAN 2015	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Volumetric muscle loss: Persistent functional deficits beyond frank loss of tissue.	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Garg, Koyal; Ward, Catherine L.; Hurtgen, Brady J.; Wilken, Jason M.; Stinner, Daniel J.; Wenke, Joseph C.; Owens, Johnny G.; Corona, Benjamin T.;	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research, JBSA Fort Sam Houston, Tx 78234	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

operative leg was aseptically prepared. A longitudinal incision was made along the lateral aspect of the leg and the skin and fascia covering the tibialis anterior (TA) muscle were bluntly separated from the musculature. The tissue covering all aspects of the ~ middle third of the tibia were separated bluntly from the bone. An osteotomy was performed approximately 5 mm proximal to the tibia-fibula junction (approximately at the midline of the TA muscle belly) using a Piezoelectric dental saw with a blade less than 0.5 mm wide. For bone stabilization after injury, a Kirschner pin (K-wire, 1.25 mm) was inserted into the medullary canal from the tibia plateau. Three-point bending mechanical testing was performed on tibias 8 weeks post-injury (Supplemental Methods).

TA Muscle VML Injury

Volumetric muscle loss was surgically created in similar fashion to that which we have previously reported.[5,6] A punch biopsy (6 mm) was performed through the middle third of the TA muscle and the biopsied tissue was removed.

In Vivo Isometric Functional Assessment

TA muscle in vivo mechanical properties were measured in anesthetized rats (isoflurane 1.5–2.0%) in both legs as previously described[5] and depicted (Sup. Fig. S1). A nerve cuff with multistranded stainless steel (Cooner Wire, 632, Chatsworth, CA) wire electrodes was implanted in each leg around the peroneal nerve. Legs were tested separately and in randomized order. The foot was strapped using silk surgical tape to a footplate attached to a dual-mode muscle lever system (Aurora Scientific, Inc., Mod. 305b, Aurora, Ontario). Optimal voltage (2–5 V) was set with a series of tetanic contractions (5–10 contractions; 150 Hz, 0.1 ms pulse width, 400 ms train). Then, the distal EDL muscle tendon and extensor hallicus longus (EHL) muscle was isolated and severed above the retinaculum. The contribution of the tenotomized EDL muscle was negligible in this testing system.[5] Peak TA muscle isometric torque was determined with the ankle at a right angle 0° and ± 20° of dorsi- or plantar flexion, assuming a moment arm of 3 mm [8] and normalized to body weight.

In Situ Isometric Length-Tension Analysis

Following in vivo functional testing, a subset of TA muscles at 4 weeks post-injury underwent in situ functional assessment of the isometric length-tension relationship, using general methodologies previously described[6] and depicted (Sup. Fig. S1). The distal 1/3 of the TA muscle was dissected free leaving the origin and neurovascular pedicle intact. The lower leg was stabilized with pins at the knee and ankle. Core body temperature was monitored and maintained at 36–37°C. The distal tendon was isolated and attached to the lever arm of a dual-mode servo muscle lever system (Aurora Scientific, Inc., Mod. 309b) and secured with 4-0 silk suture. Muscle length was measured using digital calipers. All muscles were initially set at a resting tension of ~0.2 N and performed 2–3 isometric tetanic contractions upon common peroneal nerve stimulation described above (2–5 V, 150 Hz, 0.1 ms pulse width, 400 ms train; nerve cuff as described above). The baseline tension was reduced to <0.03 N, and then the TA muscle was intermittently lengthened 0.5 mm every 90 s- pilot testing did not indicate induction of fatigue. At each stage, muscle length and active (amplitude of force) and passive tension during an isometric tetanic contraction were measured. Maximal isometric force (P_o) was determined as the peak active force produced as a function of muscle length; optimal muscle length (L_o) was defined as the muscle length at P_o.

Active isometric force normalized to P_o (%) and passive tension (N) were plotted as function of muscle length change relative L_o to determine muscle excursion and stiffness, respectively. The muscle passive length-tension curves were plotted and fitted with quintic polynomials and the active muscle length-tension curves were plotted and fitted with cubic polynomials using MATLAB® R2011a. The stiffness of the muscle was calculated as the first derivative of the passive muscle length-tension curve up to 2.5 mm longer than the optimal muscle length. Muscle excursion was defined as the change in muscle length from maximum value of P_o to the mathematically predicted 50% of maximum P_o.[9]

TA Muscle Immunohistology

Frozen sections of muscles harvested 4 and 8 weeks post-injury were probed collagen I (1:500, Millipore AB755P, Billerica, MD) and nuclei (DAPI; 1:100, Invitrogen, Carlsbad, CA) using standard methodology reported previously (Supplemental Methods) and qualitatively assessed.[6]

qRT-PCR

Gene expression was measured in cross sections of 8 week Osteotomy and Osteotomy + VML TA muscles (n = 4/group) that was comprised of the defect area and the remaining muscle mass (50–100 mg) as described previously.[5] The primer sets for TGF-ß1, connective tissue growth factor (CTGF) and ribosomal 18S are listed in Sup. Table S1.

Clinical Case

A patient's course of treatment following an open tibia fracture is presented. Methodology for torque and functional assessments are listed in the supplemental methods.

Statistics

Dependent variables were analyzed using one and two-way ANOVAs or independent samples *t*-tests. Post-hoc means comparison testing was performed when a significant ANOVA was observed. Alpha was set at 0.05. Values are listed as means ± sem. Statistical testing was performed with Prism 6 for Mac (Graphpad, La Jolla, CA).

RESULTS

Rodent Preclinical Study

Tibia Healing

In all groups that underwent osteotomy (i.e., with or without TA muscle VML injury), injured tibia had similar mechanical strength to uninjured tibia by 8 weeks post-injury (e.g., Maximal load at 8 weeks: Uninjured versus Osteotomy-only versus Osteotomy + VML; 85.5 ± 2.0, 102.2 ± 7.2, & 82.7 ± 25.8 N, ANOVA $p = 0.517$).

In Vivo TA Muscle Functional Assessment

TA muscles with VML (with or without Osteotomy) exhibited persistent and statistically similar maximal isometric tetanic torque deficits at 4 and 8 (e.g., VML + Osteotomy: 38 and 27% vs. Osteotomy) weeks post-injury in vivo at 0° of ankle flexion (Fig. 1). Neither sham-operated nor osteotomy-only groups presented TA muscle torque deficits in the involved leg. Moreover, functional deficits after VML were not

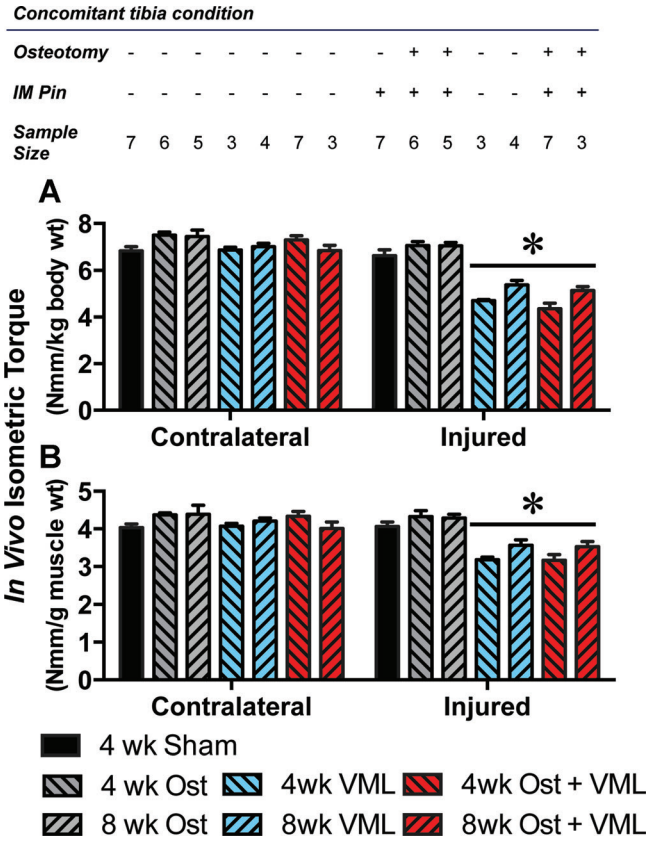

Figure 1. Volumetric muscle loss results in muscle weakness that persists after bone healing. Contralateral and affected limb TA muscle peak tetanic isometric torque (0° of ankle dorsiflexion) was measured in vivo and was expressed relative to A) body weight or B) muscle weight (lower panel). *, ≠ from respective contralateral and affected sham and osteotomy groups, $p < 0.05$. Values are means ± sem.

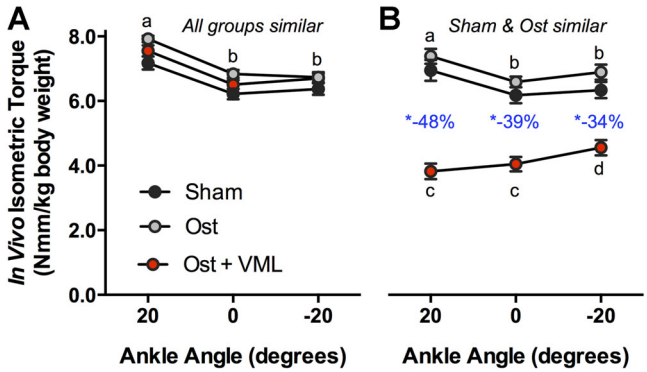

Figure 2. In vivo isometric torque deficits of VML injured muscle are dependent on joint angle. Isometric force produced by TA muscles from A) contralateral and B) involved legs was measured at 0° of ankle flexion and ± 20° of dorsi- (+) and plantarflexion (−). Values are means ± sem. For contralateral muscles, only a main effect of joint angle was observed; for all groups, values at joint angles denoted with different letters are significantly different. For injured muscles, a group x joint angle interaction was observed; values denoted with different letters are significantly different; *, deficits are calculated from osteotomy values; $p < 0.05$.

accounted for solely by the loss of muscle mass, as isometric torque 0° normalized to muscle weight still exhibited a ~23% torque deficit (Fig. 1). Notably, the similar functional deficits of VML injured muscles regardless of osteotomy likely reflects the severe nature of VML outstripping the regenerative capacity of skeletal muscle[5] – the regenerative process following other muscle injuries (e.g., crush) may be affected differently. Based on these results, only the VML + Osteotomy group were further investigated.

The joint angle-TA muscle torque relationship was investigated in vivo four weeks post-injury. TA muscle isometric torque was measured at three ankle joint angles (0° and ±20° of plantar and dorsiflexion). At all joint angles, VML resulted in a significant functional deficit, although a greater torque deficit was observed in dorsiflexion (Fig. 2). Interestingly, the torque-angle relationship was significantly different among control group and VML injured muscles. In sham and osteotomy muscles, torque measured in dorsiflexion was greater than for plantarflexion, but for VML injured muscle the opposite relationship was observed (i.e., dorsiflexion < plantarflexion). Thus, it would appear that (i) the rat TA muscle operates in vivo on the plateau to descending limb of the length-tension curve, as is expected since optimal sarcomere length in the rat is 2.4 μm[10] and measured sarcomere length in the TA muscle at the extremes of in vivo range of motion are ~2.58–3.04 μm,[11] and that (ii) VML shifts the operating range towards the ascending limb.

In Situ TA Muscle Functional Assessment

TA muscle in situ functional analyzes were performed after the in vivo functional assessment in a subset of rats four weeks post-injury. Active maximal isometric tetanic force (P_o measured at L_o) was significantly reduced (−32%) in VML injured muscles. Production of force in vivo at all angles and in situ at L_o were significantly correlated among experimental groups ($R^2 = 0.92$, $p = 0.041$; Sup. Fig. S2). However, L_o was significantly longer for VML injured muscles (Fig. 3), indicating a rightward shift of the length-tension relationship. To further investigate the impact of VML on length-tension properties of the TA muscle, active tetanic isometric torque was normalized to P_o and plotted against muscle length change relative to L_o (Fig. 4A & B). Analysis of the normalized relationship indicated a significant reduction (~31%) in functional excursion of VML injured muscle compared to sham-operated and osteotomy-only control muscles. In addition to active force, passive isometric tension (N) as a function of muscle length change was also investigated (Fig. 4C & D). In comparison to control group muscles, VML muscles began to noticeably increase passive tension in close proximity to L_o and were significantly stiffer as the muscle was lengthened.

Histological and Molecular Analyzes

Qualitative analysis of tissue histology indicated greater collagen I deposition after VML (Fig. 5A); And,

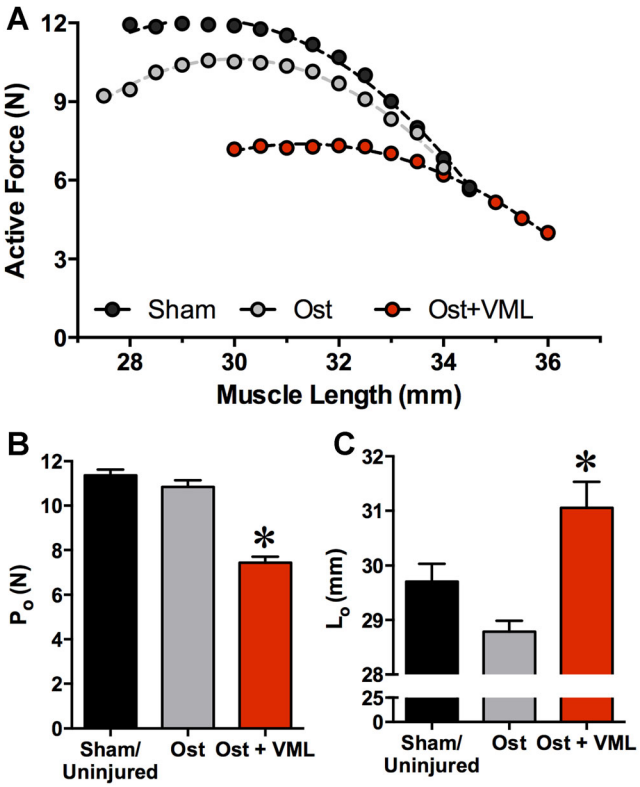

Figure 3. VML reduces maximal force and shifts optimal length to longer muscle lengths in situ. A) Representative length-tension relationships for TA muscles are presented. B) Maximal force (P_o) was significantly reduced and C) muscle length at which P_o was measured (L_o) was significantly increased with concomitant VML, $^* p < 0.05$. Values are means ± sem.

TGF-ß1 and CTGF expression, genes involved in extracellular matrix protein production, were significantly up-regulated after VML (Fig. 5B).

Clinical Case Report

Our patient is a 23 year-old soldier who sustained a type III open tibia fracture from a mounted IED blast. As a result of the blast injury, he sustained volumetric muscle loss of both the anterior and posterior compartments of the involved leg. He underwent limb salvage, which consisted of multiple debridements followed by placement of a ringed external fixator eight days following his injury. Due to some soft tissue loss, he also required split thickness skin grafting two weeks post-injury. Sensory function in the tibial, sural, saphenous, and superficial and deep peroneal nerve distributions were intact. He went on to nonunion, which required posterolateral bone grafting 11 months following his injury. His ringed external fixator was removed four months later (15 months post-injury) after he healed his tibia fracture, which was confirmed on both radiographic and clinical exam (Fig. 6).

Approximately 18 months post-injury, the patient demonstrated reduced functional capacity during standardized functional tasks (Sup. Table S2). During isometric (Fig. 6) and isokinetic testing (Sup. Fig. S3), dorsi- and plantarflexor-torque deficits (34–100%) were observed in the involved limb. The magnitude of isometric torque deficits for the plantarflexors was dependent on joint angle, although at the angles tested for the dorsiflexors, this relationship was not observed. Palpation of the anterior compartment of the involved limb during isometric contraction at 45° of plantarflexion indicated muscle activation; however, fibrous tethering appeared to restrict torque production at this joint angle. During isokinetic testing, the active range of motion (ROM) in the involved limb was reduced by 5.5° (Sup. Fig. S3). Passive ROM of the uninvolved ankle is 15° of dorsiflexion to 65° of plantarflexion, a total arc of motion of 80°; the involved ankle ROM was −10° dorsiflexion (lacking 10°) to 55° plantarflexion (lacking 10°), for a total range of motion of 45°.

DISCUSSION

The most important finding of this study is that the volumetric loss of muscle concomitant with fracture results in persistent muscle functional deficits – the magnitude of which is dependent on joint angle and muscle length. It is intuitive and has been shown previously in humans[12] and rodents[5] that volumetric muscle loss persistently reduces maximal strength; however, less intuitive is the impact of VML on maximal strength as a function of joint angle. In isolation, skeletal muscles exhibit an inverse parabolic relationship between maximal isometric force and muscle length.[13] In vivo, muscles tend to work on either the ascending or descending limb of this relationship.[10,14] Herein, we observed that uninjured rat TA muscles operate on the plateau to descending limb of the isometric torque-joint angle relationship, which is consistent with the reported TA muscle sarcomere length range during ankle motion in vivo.[10,11] In contrast, VML injured rat TA muscles appear to operate on the ascending limb of the joint angle-torque relationship. The implication of these findings is that strength deficits may be significantly worse at joint angles in which the muscle is in a shortened position (i.e., dorsiflexion for the TA muscle).

A notable observation made in situ was that the functional excursion of the muscle was reduced by ~one-third of its normal range. Since muscle excursion is an index of fiber length,[15] it is interesting to speculate that VML results in a persistent reduction in fiber length. Assuming a fiber length to muscle length ratio of 0.57 in uninjured rat TA muscle (*i.e., an individual fiber runs ~60% of the length of the muscle)*,[16] the majority of the muscle's fibers were likely shortened (*either uni- or bi-sected)* at the time of injury in this VML model (Sup. Fig. S4). It is currently unclear how a VML injured muscle adapts to this initial injury, in particular on the sarcomere level. Both sarcomere number and working length are adaptable to changes in loading due to tenotomy or tendon transfer,[17,18] and these adaptations are still

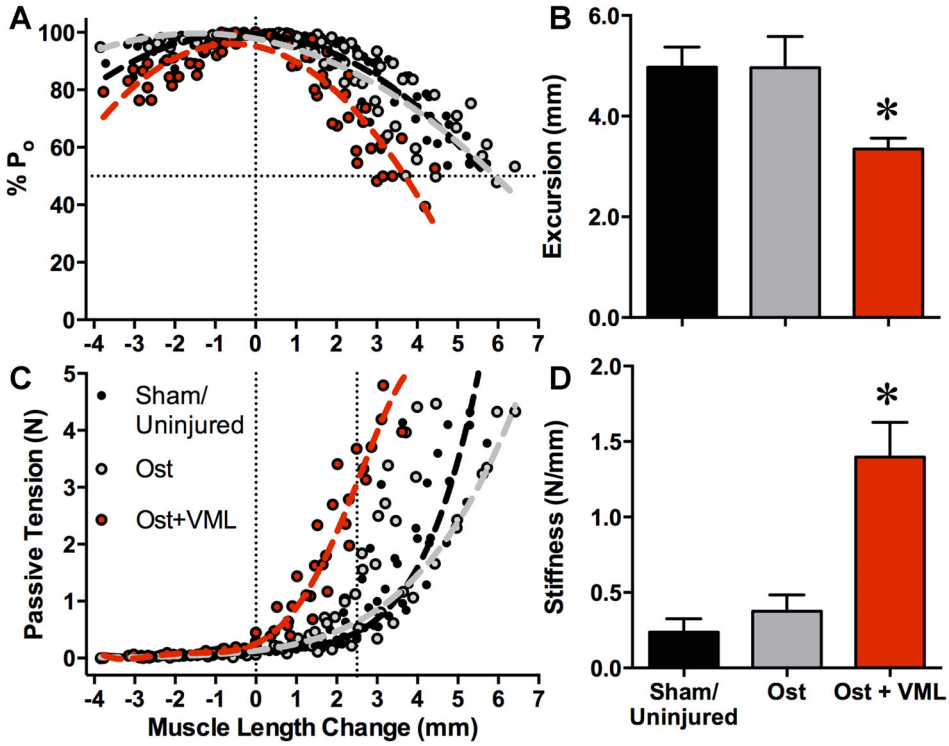

Figure 4. VML reduces functional excursion and increases muscle stiffness. A) In situ TA muscle isometric tetanic active force was measured as a function of muscle length and normalized to maximal force (P_o). B) Muscle excursion (muscle length change to 50% P_o) was significantly reduced in muscle with VML, * $p < 0.05$. C) Passive tension was normalized about L_o for each length-tension curve, and D) muscle stiffness over a length change of +2.5 mm was significantly greater with VML,* $p < 0.05$. Values are means ± sem.

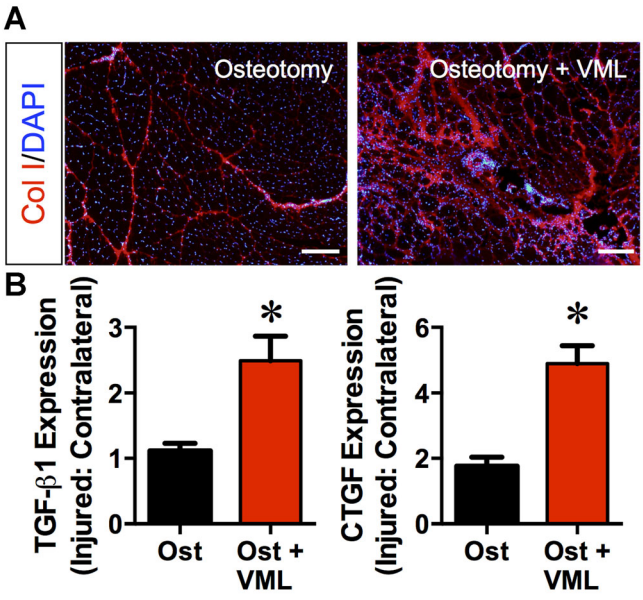

Figure 5. VML induces intramuscular fibrosis. A) TA muscles were immunostained for Collagen I at 4 and 8 weeks post-injury (*only one representative image at 8 weeks is presented due to tissue phenotypes of each group being similar at each time point*). B) Gene expression of TGF-ß1 and connective tissue growth factor (CTGF) was measured in TA muscles harvested 8 weeks post-injury. Values are means ± sem; * $p < 0.05$.

being understood for complex injuries and clinical conditions.[9,19] Although the geometry of VML defects is not uniform in the clinic, further investigation of muscle architectural modifications after VML may improve our understanding of the pathophysiology of the injury and of the design constraints for emerging tissue-engineering therapies.

Muscle injuries often result in increased extracellular matrix content. We are not aware of a histological characterization of human muscle with chronic VML; however, in rat TA muscles, VML has been shown previously[5,6] and herein to promote extensive deposition of intramuscular collagen, prolonged up-regulation of pro-fibrotic genes, and a significant increase in passive muscle stiffness. Previously, extracellular matrix deposition in the VML defect was reported to attenuate prolonged cycles of degeneration and regeneration in the remaining muscle and therefore may be a protective mechanism in this injury.[6] There are other examples in the literature indicating "fibrosis" as a positive adaptation to altered mechanical loading.[20] Future studies are needed to determine if antifibrotic therapies are beneficial to the prolonged outcome of VML injured muscle.

The clinical case highlights a common occurrence of successful limb salvage after severe open tibia fracture where the patient demonstrates persistent neuromuscular functional deficits. Though achievement of tibia union was not without complication, orthopedic surgical

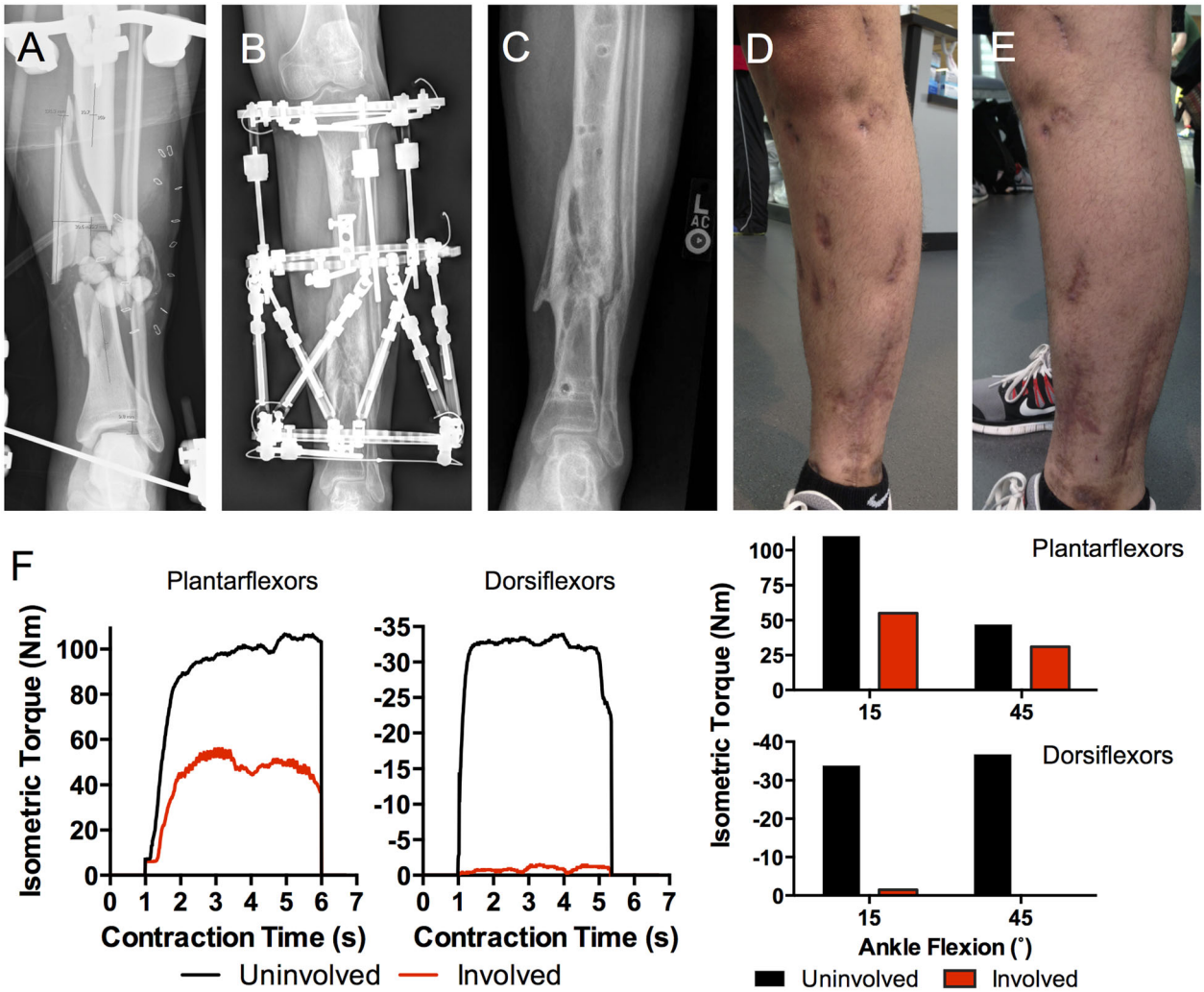

Figure 6. VML promotes persistent muscle functional deficits after tibia union following open type III fracture. A–C) Open tibia fracture was resolved approximately 15 months post-injury. D & E) Volumetric muscle loss was visible in the anterior and posterior compartments of the lower leg of the involved limb. F) Isometric plantar and dorsiflexor torque was assessed in the uninvolved and involved limb at two angles of dorsifexion (0° is neutral) using a Biodex.

care and physical rehabilitation salvaged the injured limb. However, the muscle tissue lost in both the anterior and posterior compartment of the left lower leg did not regenerate, and therefore resulted in ~34–100% strength deficits of the dorsi- and plantar-flexors 1.5 years after injury. In addition to persistent strength deficits, our patient presented with other functional deficits, which were predicted from the rodent model. Notably, strength deficits demonstrated some dependency on joint angle, and during isokinetic testing, the involved limb's functional range of motion was noticeably abbreviated. However, an important distinction in our patient is the development of musculocutaneous tethering and reduced passive range of ankle motion; and therefore multiple factors in addition to muscle weakness likely contribute to diminished limb function. To this end, it is anticipated that high-energy trauma, as presented in our clinical case, would likely exacerbate the soft tissue pathophysiology in the "open fracture" animal model.[21]

There remain many knowledge gaps in our understanding of the pathophysiology of VML. As a result, current physical rehabilitation and developing regenerative medicine (e.g., tissue engineering) therapies for VML may be further optimized. This study highlights the complexity of VML injury: Not only the gross loss of muscle tissue but also architectural and histologic adaptations of the remaining tissue underlie the persistent functional deficits. Therefore, approaching full strength recovery of VML injured muscle will require therapies that maximize the functional capacity of the remaining muscle tissue, promote de novo regeneration of muscle tissue, and integrate these tissues to re-establish the intrinsic properties of the original muscle.

ACKNOWLEDGMENTS

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Departments of the Army or

Defense. This work was supported by the Combat Casualty Care Research Program.

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